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IDENTIFICATION OF NOVEL SEX-SPECIFIC PCR-BASED MARKERS TO DISTINGUISH THE GENDERS IN EGYPTIAN DATE PALM TREES

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ABSTRACT

Date palm (*Phoenix dactylifera* L, 2n=36) is outstanding for the economic value of its different parts and its socio-economic significance in agro-systems of arid zones. Date palm trees are propagated either from seed or vegetative off shoots. Propagation with seeds is unsuitable for commercial production because half of the progeny are males and to date there is no way to distinguish the sex in date palm plants at an early stage of development. During the past decade, there have been numerous attempts to use molecular markers to discriminate among male and female trees in date palm. Here, we employed an effective approach to develop sex-specific PCR-based markers to distinguish the genders in Egyptian date palm. A set of 23 SCoT and 122 RAPD primers were applied against five superior Egyptian date palm cultivars to identify any sex-specific markers. Two SCoT (SCoT36 and SCoT41) and four RAPD primers (OP-A11, OP-M11, OP-O07 and OP-S07) exhibited differential fragments/bands between males and females (Two male-associated markers and five female-associated markers). To verify these results, the PCR reactions were repeated at least three times by different professional hands and by using different commercial reagents (Promega, Ferments and Takara). Then, these differential bands were extracted, cloned to pGEM-T Easy Vector System and transformed into DH5-alpha *E. coli* competent cells for subsequent sequencing analysis. The BLAST analysis results indicated that SCoT36 revealed high degree of similarity with mitochondrial *rpl*2 gene in date palm while, SCoT41 revealed high degree of similarity with putative DEIH-box RNA/DNA helicase gene (Os01g0767700) in *Oryza sativa* Japonica. On the other hand, the BLAST analysis of (OP-A11, OP-O07 and OP-S07) revealed no significant similarity to any well-defined sequences or protein on databases. While, they showed very low similarity with the date palm published sequences which give high probability that this sequences may be a novel or non-sequenced parts of the date palm genome. Meanwhile, OP-M11 exhibited high degree of similarity with the date palm genome (cultivar Deglet Noor, fosmid 9B12) and partial similarity with both putative glucuronoxylan glucuronosyltransferase protein (*Triticumurartu*) and Exostosin-like family protein (*Oryza sativa*). These results represent the first case-study focusing on the applications of SCoT technique as a gene targeting marker in sex-determination in date palm. Moreover, indicate that these promising primers should be helpful in rapidly distinguishing between male and female trees in date palm at earliest stages.

KEYWORDS: Date Palm, Sex-Determination, SCoT, RAPD, Molecular Markers

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is a dioecious perennial tree with a great socio-economic importance especially in the Middle East, North Africa and to a small extent in California and Mexico. The date palm trees are cultivated not only for their valuable fruits (dates), but also for producing fuel, fiber and as shelter for ground crops. According to recent records, there are about 100 million date palm trees exist around the world with the vast majority

located in the Middle East and North Africa. Date palm is a high salt-tolerant tree and may harbor a good production even under 3,000 ppm of salty water. Some varieties had exhibited a high tolerance of total dissolved salt (22,000 ppm) but their productivity had been affected.

Date palm is an important economic crop in Egypt where the world's largest producer over the last two years is Egypt with 1,470,000 mt followed by Islamic Republic of Iran (1,066,000 mt) and Saudi Arabia (1,050,000 mt) (FAOstat, 2012). The establishment of date palm orchards is a long term and costly investment. Since, the date palm species is commonly propagated by offshoots and by seeds. For both approaches, extremely slow growth of seedlings and offshoots does not allow the use of classical breeding techniques; it takes 8–10 years before plants produce fruit. Moreover, healthy date palm tree usually produce from 0 to 3 offshoots per year and, in general, not more than 10–40 during its lifetime depending on the cultivar and the environmental conditions. Although, seed germination is the easiest but seedlings may take up to 10 years before flowering and fruiting. Usually the progeny derived from seeds are heterozygote and do not carry the same mother characters. Nevertheless, such propriety of sexual propagation is intended to create new genotypes and provides a basis for the selection of elite trees. Moreover, offspring from seeds are approximately equally split between males and females; however, many fewer male plants as pollen donors are generally required as compared with female plants (one male is used to hand pollinate about 90–100 females) (Bounaga 1993).

In all date palm, dioecy presents a challenge in their breeding programs because it is impossible to distinguish trees gander until they flower approximately 5 to 8 years after planting (Aberlenc-Bertossi *et al.,* 2011; Bendiab *et al.,* 1993). The conventional technique such as the morphological screening could be applicable, but particularly when the fruits are ripe. Date palm is cytogenetically recalcitrant material, having tiny and sticky chromosomes (Soliman and Al-Mayah 1978 and Al-Salih *et al*., 1987). Although, *Phoenix dactylifera* (date palm) is one of a number of dioecious members of the Palmae but is the only palm species with authenticated sex chromosomes, probably of the XX/XY type. The development of a cytological method based on chromocyanin staining has also shown the occurrence of sexual chromosomes carrying distinctive nucleolar heterochromatin (Siljak-Yakovlev *et al.,* 1996). On the other hand, studying the genomic size of date palm using flow cytometry and Arabidopsis as a standard, Ouenzar *et al*. (2001) had estimated the 2X DNA to 490 Mbp (0.51 pg).

Despite increasing research efforts on a number of different plant species, there is relatively little information available on the molecular basis of sex determination and it is even difficult to estimate the numbers of genes involved, particularly as the genes which result in organ suppression are unlikely to be the primary sex determining genes. In the last two decades, there have been serious efforts to understand the basis of sex determination in date palm and to develop methods of identifying the gender at an early stage using isozymes (Torres and Tisserat, 1980), peroxydases (Majourhat *et al*., 2002), molecular marker tools using random amplified polymorphic DNA (RAPD) (Younis *et al.*, 2008) and PCR-based restriction fragment length polymorphism (PCR-RFLP) approach (Al-Mahmoud *et al.*, 2012).

In this paper, for the first time, we employed two marker systems including a novel gene targeting marker (SCoT) and Random Amplified Polymorphic DNA (RAPD) in an attempt to identify sex-specific markers in Egyptian date palm at early stage.

MATERIALS AND METHODS

Plant Material

The samples of superior date palm cultivars were collected from each gander as following:

- **For RAPD analysis**

Ten samples representing the male and female of five Egyptian cultivars were subjected to molecular analysis.

- **For SCoT analysis**

Twelve samples representing the male and female of three Egyptian cultivars (two plants from each gender) were subjected to molecular analysis. Two additional samples were prepared to represent bulked males and bulked females.

High-quality genomic DNA was extracted from fresh leaves (100 mg) of all collected plants using a DNA easy Plant Mini Kit (QIAGEN, Santa Clarita, CA) and according to the manufacturer's protocol.

Random Amplified Polymorphic DNA (RAPD) Analysis

RAPD-PCR was carried out as described by Diab *et al*, (2013). A set of 122 random 10-mer primers were applied against the ten date palm samples in order to identify any sex-specific markers.

Start Codon Targeted Polymorphism (SCoT) Analysis

SCoT-PCR was performed as described by Adawy *et al*, (2013). A set of 23 18-mer primers were used against the twelve date palm samples in order to identify any sex-specific markers.

Cloning of the PCR Product and Transformation

The fragments that exhibited differential pattern between males and females from both techniques (RAPD and SCoT) were extracted from agarose gel using QIAquick Gel Extraction Kit (QIAGEN, Santa Clarita, CA). Then the purified PCR products were cloned to pGEM®-T Easy Vector System (Promega) according to the manufacturer's instructions and transformed to DH5-alpha *E. coli* competent cells for subsequent sequencing analysis.

Positive transformants /colonies were verified using PCR approach. Then plasmid miniprep was performed of positive clones to harvest the vectors potentially with insert using QIAprep® Miniprep purification kit (QIAGEN, Santa Clarita, CA).

DNA Sequencing Analysis

Sequencing of candidates of pGEM®-T Easy Vector with SCoTs and RAPDs inserts were accomplished with an ABI310DNA sequencer (Applied Biosystems, Norwalk, Conn.) by using synthetic primer complementary to the vector sequences flanking the multiple cloning sites (M13 forward primer). To confirm that each purified PCR product not contain more than one sequence with the same molecular size; eight verified positive clones were subjected for sequencing analysis. The sequence reads were aligned, using ClustalW multiple sequence alignment algorithm (Larkin *et al.*, 2007), against the Qatari date palm genome. BLAST analysis (Altschul *et al.*, 1990) was used to identify the regions among the reads that were not well aligned with the reference sequence (or the vector backbone) by an NCBI DNA sequence database search. A homology search was performed using BLASTX against the NCBI protein database (http://www.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSIONS

- **SCoT Analysis**

Out of twenty-three 18-mer primers were applied against the twelve date palm male and female samples in order to identify any sex-specific markers, two SCoT primers (SCoT36 and SCoT41) successfully revealed a differential amplicons can distinguish between males and females of the three Egyptian cultivars (Figure 1). The SCoT's gels pattern revealed that the two SCoT primers exhibited only one differential band present in all females and its bulk, while absent in all males and its bulk. To verify SCoT results, these two primers were repeated at least three times by three different professional hands and by using different commercial reagents (Promega, Ferments and Takara).

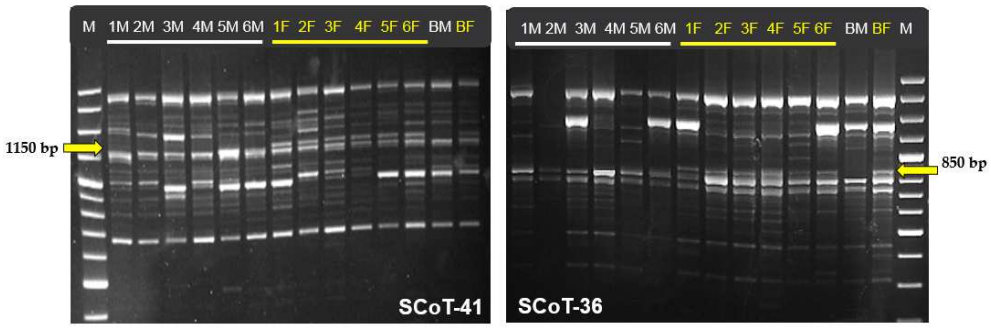

Figure 1: SCoT-PCR Patterns of the Date Palm Collected Samples as Revealed by Primers SCoT-36 and SCoT-41. Samples: 1M= Male Zaghloul-1, 2M= Male Zaghloul-2, 3M= Male Hayani-1, 4M= Male Hayani-2, 5M= Male Samany-1, 6M= Male Samany-2, 1F= Female Zaghloul-1, 2F= Female Zaghloul-2, 3F= Female Hayani-1, 4F= Female Hayani-2, 5F= Female Samany-1 and 6F= Female Samany-2. BM= Bulk of Males and BF= Bulk of Females. M: is the Standard DNA Marker 100 bp Ladder

In this context, only few studies were conducted using SCoT markers to evaluate the genetic relationships in rice (Collard and Mackill, 2009), mango (Luo *et al.*, 2010), peanut (Xiong *et al.*, 2011) and Chinese sugarcane (Que *et al.*, 2014). Therefore, the current investigation represents the first case-study focusing on the applications of SCoT technique in sex- determination in dioecious plants generally and in date palm particularly.

Sequence Analysis of SCoT Differential Isolated Markers

The two SCoT differential isolated fragments (SCoT36 and SCoT41) were sequenced then the vector sequence were removed to obtain a final approximately sequences length 910bp and 1135bp, respectively. Eight white/positive colonies were selected randomly for sequencing. Alignment of obtained sequences showed that each differential isolated fragment (SCoT36 and SCoT41) contain only one sequence per fragment.

Mega BLAST, discontiguous-MegaBLAST, BLASTn and BLASTx were used to identify if these two sequences/fragments already exists in a public database (Genbank or Protein Data-Bank "PDB"). The BLASTx analysis results generally indicated that sequence of SCoT36 revealed high degree of similarity with ribosomal protein L2 (mitochondrion - *Phoenix dactylifera*, query cover=76%, E-value= 6e-161, sequence ID: gb:YP_005090371.1), ribosomal protein L2 (mitochondrion - *Liriodendron tulipifera*, query cover=76%, E-value= 4e-136, sequence ID: gb:YP_007905729.1), ribosomal protein L2 (*Ferrocalamus rimosivaginus*, query cover =77%, E-value= 3e-151, sequence ID: gb:AEK66742.1) and 60S ribosomal protein L2 (mitochondrial - *Oryza sativa* Japonica, query cover=62%, E-value= 2e-94,sequence ID: sp|P92812.2|RM02_ORYSJ) (Figure 2). In this context, Adams *et al*, (2001) reported that the mitochondrial ribosomal protein (*rpl*2) gene can transfer in partial or complete form to the nucleus in some eudicot and monocot plants. This partial or complete missing of mitochondrial *rpl*2 gene and its transfer/movement to the nucleus was

also reported in rice (Kubo *et al*., 1996), Arabidopsis (Unseld *et al.,* 1997) and Wheat (Bonen *et al*., 1998).

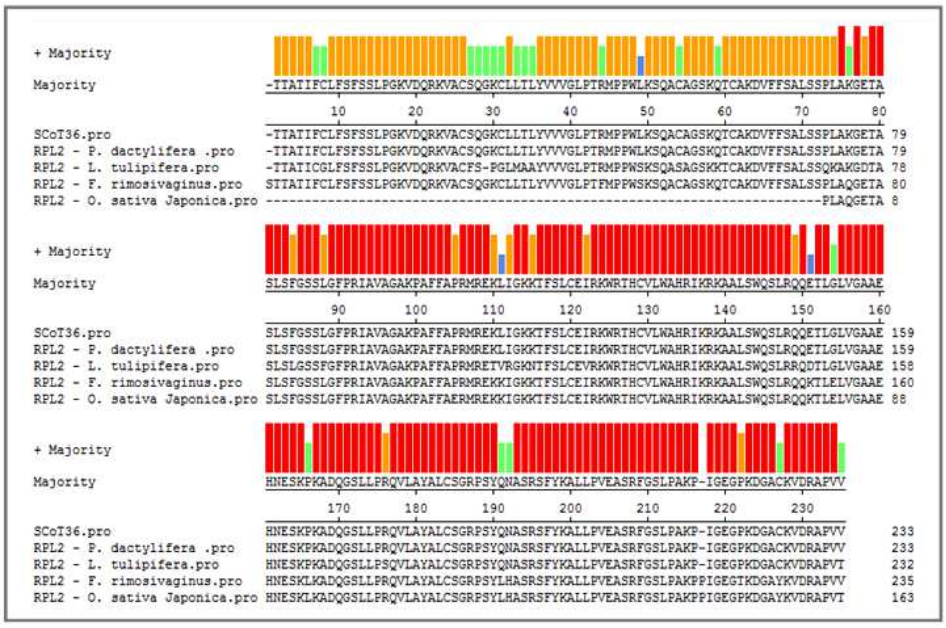

Figure 2: The BLASTx Results of the SCoT36 Sequence Showing a High Degree of Similarity (62-76% of the Total Query Length) with Mitochondrial Ribosomal Protein L2 (*rpl2*) in Different Plants

On the other hand, the BLASTn analysis results of SCoT41revealed that there are high degree of similarity with Putative DEIH-box RNA/DNA helicase (Os01g0767700) in *Oryza sativa* Japonica (query cover= 69%, E-value= 2e-167, sequence ID: gb:NM_001050897.1), hypothetical protein OsI_03881 in *Oryza sativa* Indica Group (query cover= 69%, E-value= 5e-134, sequence ID: gb:EEC71544.1), predicted ATP-dependent RNA helicase DHX36-like protein in *Oryza brachyantha* (query cover= 69%, E-value= 4e-133, sequence ID: gb: XP_006646361.1) and hypothetical protein SORBIDRAFT_03g035700 in *Sorghum bicolor*] (query cover= 69%, E-value= 2e-132, sequence ID: gb: XP_002456402.1) (Figure 3).

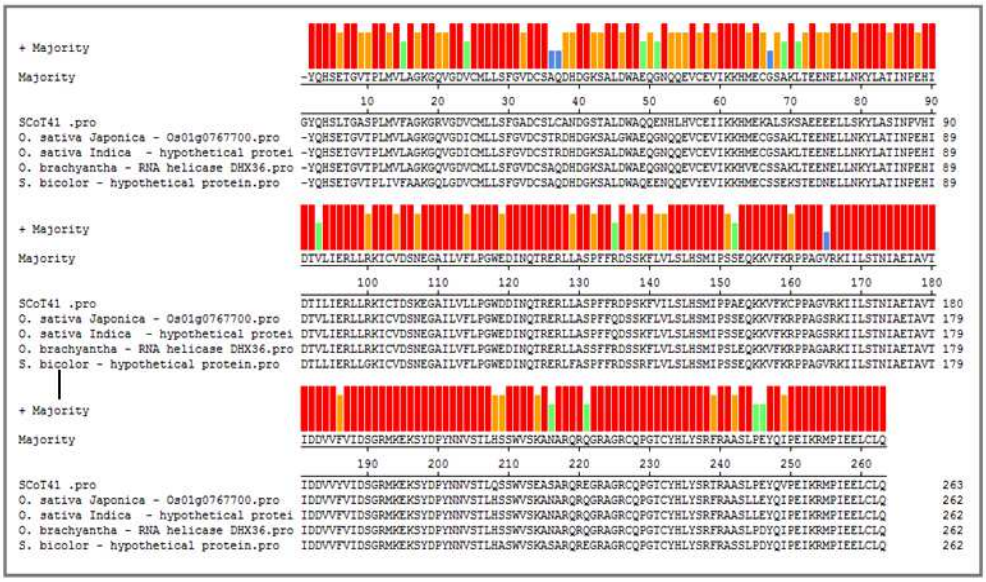

Figure 3: The BLASTx Results of the SCoT41 Sequence Showing a High Degree of Similarity with Putative DEIH-Box RNA/DNA Helicase Protein in Rice and Hypothetical Proteins in Other Plants

- **RAPD Analysis**

Out of 122 random 10-mer primers were applied against the ten date palm male and female samples in order to identify any sex-specific markers, four operon RAPD primers (OP-A11, OP-M11, OP-O07 and OP-S07) successfully generated a differential bands/amplicons can distinguish between males and females of the five Egyptian cultivars (Figure 4). The gels patterns demonstrate that OP-M11 and OP-S07 exhibited only one differential band present in all females and absent in all males. While, OP-O07 revealed only one differential band present in all males and absent in all females. On the other hand, OP-A11 showed two differential bands, the first band present in all males and absent in all females while, the second band vice versa. To verify these results, these primers revealed sex-specific markers were repeated at least three times by three different professional hands and by using different commercial reagents (Promega, Ferments and Takara).

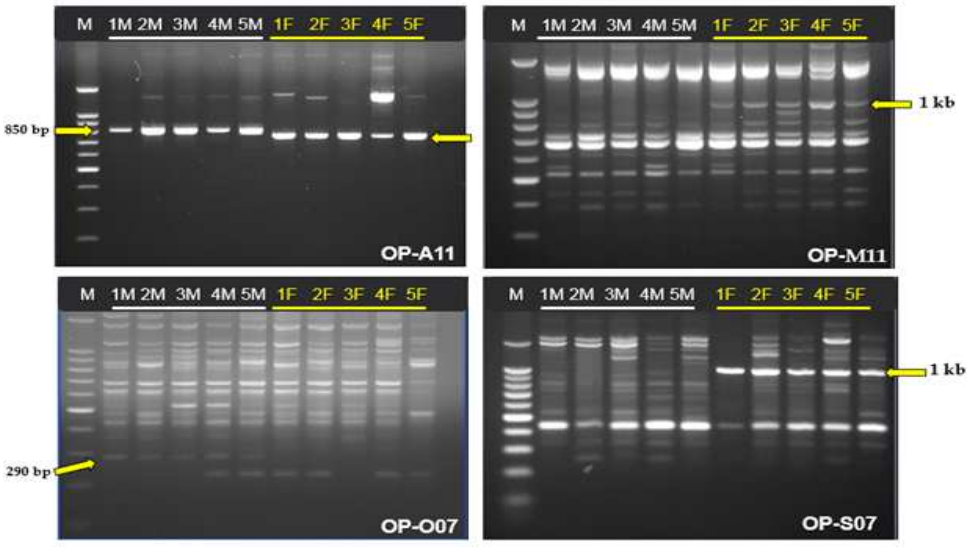

Figure 4: RAPD-PCR Patterns of the Date Palm Collected Samples as Revealed by Primers OP-A11, OP-M11, OP-O07 and OP-S07. Samples: 1M- Male Bertamoda, 2M- Male Hayani, 3M- Male Samany, 4M- Male Siwi, 5M- Male Zaghloul, 1F- Female Bertamoda, 2F- Female Hayani, 3F- Female Samany, 4F- Female Siwi and 5F- Female Zaghloul. M: is the Standard DNA Marker 100 bp Ladder

Two of the most serious limitations of RAPD markers are: (1) Repeatability in many systems, especially when transferring between populations or laboratories (Liu *et al.,* 1994). (2) Reproducibility. Wolff *et al,* (1993) reported several factors that influence the reproducibility of RAPD reactions such as: quality and quantity of template DNA, PCR buffer, concentration of magnesium chloride, primer to template ratio, Taq DNA polymerase brand or source, and thermal cycler brand.

This concerns about reproducibility and repeatability of RAPD markers, could be overcome through choice of an appropriate DNA extraction protocol to remove any contaminants, by optimizing the parameters used, by scoring only of the reproducible DNA fragments, by using appropriate DNA polymerase brand, and by repeat the RAPD-PCR many times by different professional hands in different laboratories using different commercial reagents.

RAPD banding patterns have been used to identify sex-linked markers in *Phoenix dactylifera* (Younis *et al.,* 2008), *Hippophae rhamnoides* (Persson and Nybom, 1998), Asparagus (Jiang and Sink, 1997), *Piper longum* (Bannerjee *et al.,* 1999), *Silene latifolia* (Zhang *et al.,* 1998), *Cannabis sativa* (Sakamoto *et al.,* 1995), *Actinidia chinensis* (Harvey *et al.,* 1997) and *Atriplex garettii* (Ruas *et al.,* 1998).

In this context, Younis *et al,* (2008) used only seven RAPD primers in an attempt to identify sex-specific markers in date palm (*Phoenix dactylifera*). They screen seven RAPD primers against three male and four female date palm samples represent only four dry date palm cultivars (Sakoty, Bertmoda, Malkabi, Dagana). They reported that only three primers (A10, A12 and D10) revealed female-specific markers, (490, 750 and 800 bp, respectively). While, primers (A12 and D10) exhibited two male-specific markers (370 and 675 bp, respectively).

In this investigation, we covered a broad range and types of Egyptian date cultivars (Five cultivars include dry, semi-dry and soft cultivars). Also, we used a large initial no. of RAPD primers (122 primers) comparing with those used by Younis *et al,* (2008) which used only 30RAPD primers to achieve the same attempt. Finally, we used in this investigation an effective approach to overcome the most two serious limitations of RAPD markers (Repeatability and Reproducibility) by repeat the promising RAPD primers (OP-A11, OP-M11, OP-O07 and OP-S07) many times by different professional hands in different systems using different commercial reagents in order to verify their results.

Sequence Analysis of RAPD Differential Isolated Markers

In order to identify if each one from differentially isolated fragments/bands (OP-A11, OP-M11, OP-O07 and OP-S07) carry one or more than one sequence, eight white colonies were selected randomly for sequencing. The generated sequences length after remove the vector sequence were 810bp, 940bp, 290bp and 920bp, respectively. The sequences alignment showed that three differential isolated fragments (OP-A11, OP-O07 and OP-S07) contain only one sequence per fragment. The eight sequences of differential isolated fragment (OP-M11) showed that these fragment contain two sequences (Seven colonies carry the same sequence and one carry another sequence).

The best way to identify an unknown sequence is to see if that sequence already exists in a public database (Genbank). Mega BLAST, discontiguous-Mega BLAST, and BLASTn all were used to accomplish this goal. Moreover, the sequences were also subjected to the BLASTx analysis which compares translational products of the nucleotide query sequence to a protein databases. The BLAST analysis results generally indicated that sequences of (OP-A11, OP-O07 and OP-S07) revealed no significant similarity to any well-defined sequences or protein on databases. While, they showed particularly very low similarity (less than 6-9%) with the date palm published sequences. This results giving high probability that this sequences may be a novel or non-sequenced parts of the date palm genome. On the other hand, the BLASTn analysis results of OP-M11-1 (first sequence; represented by 7 sequences with 100% similarity) indicated that there are high degree of similarity (74%, E-Value= 2e-132) with the date palm (*Phoenix dactylifera* - cultivar Deglet Noor, fosmid 9B12 - gb|JF313260.1|) (Figure 5).

Meanwhile, the BLASTx analysis result of OP-M11-2 (second sequence) indicated that there are a partial similarity (9% of the total length) with putative glucuronoxylan glucuronosyl transferase protein (*Triticumurartu*), Exostosin-like family protein (*Oryza sativa* Japonica, Sequence position=chr3:3957004-3961813), uncharacterized protein (*Oryzabrachyantha*) andpredicted protein (*Hordeumvulgare*) (Figure 6).

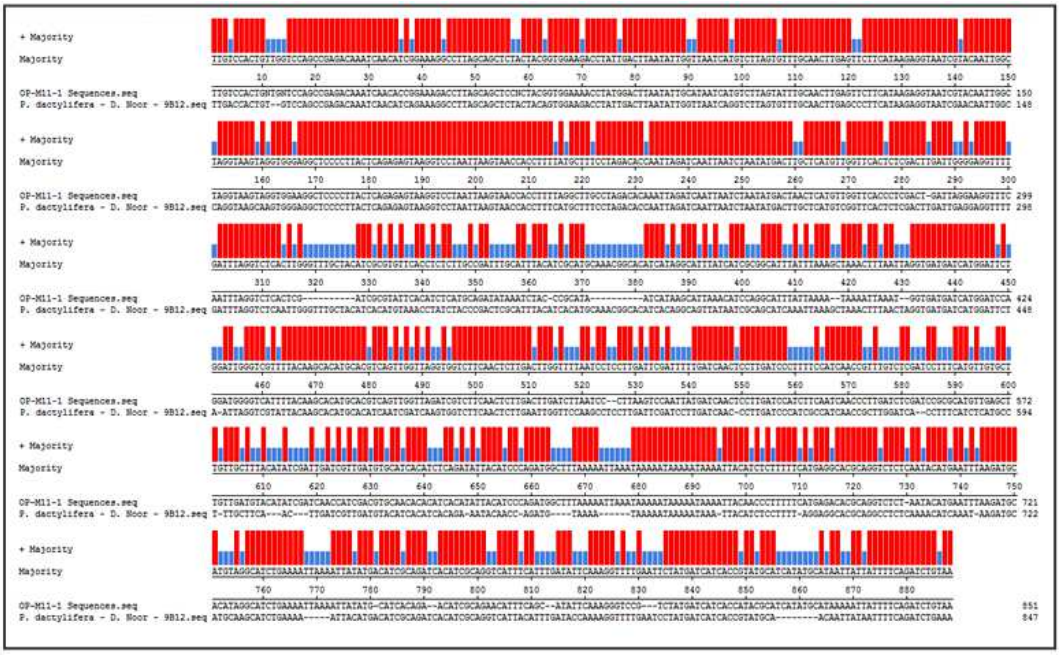

Figure 5: The BLASTn Results of the OP-M11-1 Sequence Showing High Degree of Similarity (74%, E-Value= 2e-132) with the Date Palm Cultivar Deglet Noor (Fosmid 9B12 - gb|JF313260.1|).

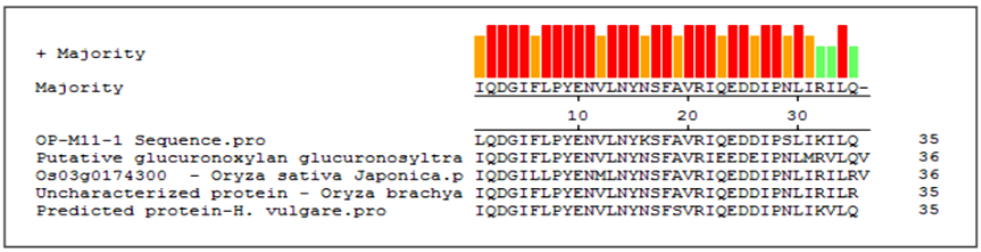

Figure 6: The BLASTx Results of the OP-M11-2 Sequence Showing a Partial Similarity (9% of the Total Query Length) with Identified or Predicted Proteins in Different Plants

In plants such as *Phoenix dactylifera*, *Silene latifolia* and *Cannabis sativa*, it is not surprising that male-associated markers are relatively abundant. In dioecious plants where sex chromosomes have not been identified, markers for male-ness indicate either the presence of sex chromosomes which have not been distinguished by cytological methods or that the marker is tightly linked to a gene involved in sex determination. Female-associated molecular markers have been described in Actinidia (Harvey *et al.*, 1997) and *Salix viminalis* (Alstrom-Rapaport *et al.*, 1998). These might arise as a consequence of close linkage with a female sex determining gene or might indicate a sequence on the X chromosome inherited from the male parent. One male is used to hand pollinate about 90–100 females (Bounaga, 1993), and for centuries emphasis has been on clonal propagation of females. This reduces the genetic diversity of the cultivars, accelerating vulnerability to biotic and abiotic stresses.

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